IN THE UNITED STATES PATENT AND TRADEMARK OFFICE {PRIVATE }

In re Application of:	Harold Trick		
Serial No.:	10/616,390	Group No.:	1638
Filed:	07/09/2003	Examiner:	Ibrahim
Entitled:	**COMPOSITIONS AND METHODS FOR CONTROLLING PARASITIC NEMATODES**		

SECOND DECLARATION OF DR. HAROLD TRICK

EFS WEB-FILED
Commissioner for Patents
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Alexandria, VA 22313-1450

I, Dr. Harold Trick, state as follows:

1. My present position is Associate Professor, Department of Plant Pathology, Kansas State University, Manhattan, KS.

2. I am an inventor of the above referenced patent application.

3. It my understanding that the Examiner has rejected the claims as being obvious over Tobias et al. (WO 01/37654) and Mushegian et al. (WO 01/96584) in view of Zipperlin et al. (EMBO J. 20(15):3884-92 (2001). The Examiner states that while Tobias and Mushegian teach targeting genes essential for nematode growth and development and parasitism, they do not disclose well characterized embryonic lethal genes from nematodes. The Examiner then states that "It would have been obvious to one of ordinary skill in the art to use the method of controlling plant parasitic nematodes by transforming the plant with a dsRNA construct that targets endogenous nematode genes essential for nematode development and growth as taught be each of Tobias et al. and Mushegian et al., and to modify that method by incorporating one or more of the embryonic lethal genes taught by Zipperlin et al.; said embryonic lethal genes can be identified and obtained from *Heterodera glycines* using sequence information from cloned *C. elegans* orthologs known in the prior art as suggested by Tobias et al., with a reasonable expectation of success. One of ordinary skill in the art would have been motivated to use dsRNA

technology to control *Heterodera glycines* in a transgenic plant, given that use of dsRNA is more safe and effective as compared to other known methods of controlling nematodes in transgenic plants as suggested by each of Tobias et al. and Mushegian et al. Therefore, the invention as a whole was prima facie obvious.

4. With regard to motivation, a person of skill in the art would not be motivated to combine the three references because the use of dsRNA is more safe and effective as compared to other known methods of controlling nematodes in transgenic plants as suggested by the Examiner. This is an oversimplification of the issue, which requires the use of embryonic lethal genes. Thus, the real question to a person of skill in the art such as myself is not whether I would be motivated to combine the reference because dsRNA is safe and effective, but whether one of skill in the art would be motivated to use dsRNA that is specific to embryonic lethal phenotype genes.

5. The following facts, supported by the literature and my knowledge as a person of skill in the art, establish that a person of skill in the art would not be motivated to combine the references to make plant that express dsRNA specific for *H. glycines* embryonic lethal phenotype genes:

- Urwin et al., MPMI 15(8):747-752 (2002)(attached at Tab 1) discloses that dsRNA corresponding to major sperm protein, an embryonic lethal phenotype gene, had no effect on development of *H. glycines*.
- Fairbairn et al., Planta 226:1525-33 (2007)(attached at Tab 2) discloses that when nematodes feed on plants that express dsRNA that has been identified as lethal in vitro, the target gene is down-regulated but there is no lethality.
- There is a difference between *C. elegans* and *H. glycines*. *C. elegans* is a free living organism, while *H. glycines* is a parasitic organism.
- None of the references cited by the Examiner teach specific constructs for targeting embryonic lethal phenotype genes in *H. glycines* or constructs that are orally active.
- There is no guidance in the references cited by the Examiner as to what *H. glycines* embryonic lethal phenotype gene constructs should be used or how to

express those genes in a plant. At best, there is only a general guidance. Even given the disclosures references by the Examiner, substantial planning and empirical research, as disclosed in the specification of the present application, was required to identify and design constructs for expression of *H. glycines* embryonic lethal phenotype genes in plants.

- A person of skill in the art, with knowledge of the references I provide above as well as the references cited by the Examiner, would recognize that it was not predictable that a dsRNA that is lethal to *C. elegans* would be lethal to a different parasitic organism such a *H. glycines* when expressed in a plant.

6. I further declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: May 11, 2009

Dr. Harold Trick

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MPMI SPOTLIGHT*

Ingestion of Double-Stranded RNA by Preparasitic Juvenile Cyst Nematodes Leads to RNA Interference

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RNA interference is of value in determining gene function in many organisms. Plant parasitic nematodes are refractory to microinjection as a means of introducing RNA and do not show any oral uptake until they are within plants. We have used octopamine to stimulate uptake by preparasitic second stage juveniles of two cyst nematodes, *Heterodera glycines* and *Globodera pallida*. This new technique was used to facilitate uptake of double stranded RNA (dsRNA) together with fluoroscein isothiocyanate as a visual marker. Targeting cysteine proteinases did not reduce the number of parasites but caused a shift from the normal female/male ratio of 3:1 to 1:1 by 14 days postinfection (dpi). Exposure of *H. glycines* to dsRNA corresponding to a newly characterized protein with homology to C-type lectins did not affect sexual fate, but 41% fewer parasites were recovered from the plants. As expected, treatment with dsRNA corresponding to the major sperm protein (MSP) had no effect on either parasite development or sexual fate over 14 days. Northern analysis showed lower transcript abundance for the two targeted mRNAs that occur in J2, plus a later inhibition for MSP transcripts when males developed sperm at 15 dpi. These findings establish a procedure for RNAi of plant parasitic nematodes.

Nematodes show highly complex parasitic interactions with plants and cause over $100 billion of annual crop losses to world agriculture (Sasser and Freckman 1987). Cyst-nematodes such as *Globodera pallida* and *Heterodera glycines* are important examples and key pests of potato and soybean, respectively. Cyst-nematodes are obligate parasites and take several weeks to complete their life cycle. The second-stage juvenile (J2) of a cyst nematode hatches from a previously dormant egg, often following stimulation by a chemical factor diffusing from host roots. Its small size (approximately 450 μm long and 18 μm wide) enables the J2 to migrate through plant root cells from the zone of elongation to a site by the vascular tissue where feeding is initiated. The established J2 modifies the plant cell, causing progressive wall loss and a merging of cytoplasm. The resultant large syncytial cell, essential for female development, acts as a transfer cell providing nutrient flow to the now sedentary parasite. Both sexes feed as second and third stage juvenile (J2, J3) parasites and sexual dimorphism arises. The males do not feed in later stages, and they regain mobility as adults before fertilizing the sedentary, saccate female. The J3 female remains sedentary and grows at a rapid rate to reach J4 stage. A mature female is approximately 100-fold larger than the male. It is the female whose feeding causes much of the crop loss associated with these major pests. After fertilization, the female body wall tans to form a protective cyst around many of the several hundred eggs, each containing a J2. Many of this next generation can enter dormancy until another host crop is grown.

As obligate parasites with a life cycle of several weeks, cyst nematodes prove refractory to studies aimed at defining genes essential for development and plant parasitism. The full range of genetic approaches that has been deployed so incisively with the microbivorous nematode *Caenorhabditis elegans* is not readily applied to them. Fire and associates (1998) reported that RNA interference (RNAi) is a means by which double stranded RNA (dsRNA) induces sequence-specific posttranscriptional gene silencing. In *C. elegans* postgenomic studies have utilized the power of RNAi to determine gene function. RNAi has been used to systematically analyze 90% of the predicted genes on chromosome I (Frasier et al. 2000), 96% of the predicted genes on chromosome III (Gonczy et al. 2000), approximately 2,500 nonredundant cDNAs (Maeda et al. 2001), and the function of 350 oocyte cDNAs (Piano et al. 2000). These projects have rapidly advanced the understanding of gene function in *C. elegans*. The mode of action by which RNAi operates is being elucidated. Long dsRNA sequences are more active than short dsRNAs in effecting gene silencing (Sharp 2001). The long dsRNA is processed into short interfering RNAs (siRNAs) of 21 to 23 nucleotides by an RNase III-like activity. These siRNAs mediate cleavage of mRNA through sequence complementarity (Elbashir et al. 2001; Hammond et al. 2000; Zamore et al. 2000). The mode of action of RNAi has been reviewed in detail (Sharp 2001).

Fire and associates (1998) demonstrated that RNAi abolished expression of targeted genes in *C. elegans* by injecting dsRNA. Subsequently, others have shown that the effect occurs when *C. elegans* is fed the bacterium *Escherichia coli*, which transcribes the recombinant dsRNA (Timmons and Fire 1998). It also results from simply soaking the animals in dsRNA preparations (Tabara et al. 1998). In animal parasites, electroporation has been used as a means of delivering dsRNA to *Trypanosoma brucei* (Ngo et al. 1998). These modes of dsRNA delivery are not readily applied to plant parasitic nematodes. The small size and well-formed cuticle of a cyst nematode J2 make microinjection both difficult and dependent on special equipment, and this technique has yet to be routinely employed. Delivery of dsRNA by ingestion is difficult, as the nematode only feeds following the establishment of a feeding site inside the root and does not ingest substances prior

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to this stage. Here, we report a procedure that results in the uptake of dsRNA molecules by J2s, thus enabling RNAi to be applied to cyst nematodes and the approach to be used to study the effects of RNAi on three genes. The approach should help define nematode gene expression that is essential for their successful interaction with plants.

RESULTS

Uptake of fluorescein isothiocyanate (FITC) by cyst nematodes stimulated by octopamine.

Analogues of the seratogenic neurochemical 5-hydroxy tryptamine (5HT, serotonin) induce plant parasitic nematodes to release pharyngeal gland secretions (Willats et al. 1995). In *C. elegans*, a biologically active amine, octopamine, has the opposite effect of 5HT (Horvitz et al. 1982). In the expectation that the opposing effect may be true in tylenchids, we examined the possibility that octopamine may affect pharyngeal pumping and ingestion. This was demonstrated by the observation of fluorescence in the pharyngeal lumen following soaking in 50 mM octopamine and FITC (Fig. 1). The lumen of the pharynx is not autofluorescent and, in the absence of octopamine, no fluorescence can be observed because second stage preparasitic J2s do not ingest. Occasionally, in the absence of octopamine, uptake of the dye by preparasitic J2 cyst nematodes was limited to the anterior sense organs (amphids) and some of their neurones (Atkinson et al. 2001). Uptake of FITC was observed in the pharyngeal lumen and in the excretory/secretory system; entry by either route may facilitate RNAi as dsRNA has the ability to cross cellular boundaries (Fire et al. 1998).

Stages of *C. elegans* readily swallow the aqueous medium around them, and this can be monitored using the fluorescent dye FITC (Fig. 1A). Uptake of FITC by *H. glycines* and *G. pallida* was induced by the presence of 50 mM octopamine, a biologically active amine. Following treatment, FITC was present in the lumen of the mouth stylet, which is normally used to pierce plant cell walls, and is clearly passed posteriorly to the pharyngeal lumen (Fig. 1B, C). The excretory/secretory system of the parasite also showed uptake (Fig. 1D). Uptake was typically limited to 15 to 20% of individuals through the stylet but was often >50% via the excretory/secretory system. We have concentrated on studying cyst nematodes but are exploring the possibility of developing the technique for application with *Meloidogyne incognita*.

RNAi of target genes.

We have explored the potential of the new RNAi technique to deliver dsRNA molecules targeted against three very different cyst nematode genes. First, we targeted cysteine proteinases because their inhibition by specific protein inhibitors as transgenes in plant hosts affects growth of established parasites (Urwin et al. 1997a; 1998; 2001). J2 of *H. glycines*, *G. pallida*, and mature *C. elegans* were treated for 4 h with combinations of dsRNA corresponding to a cysteine proteinase from each species (*hgcp*-1, *gpcp*-1, or *gcp*-1, respectively) with or without 50 mM octopamine. FITC was included as a reporter of uptake. The relative transcript abundance immediately after treatment was determined by virtual Northern analysis (BD-Clontech, Oxford). All the cDNA was electrophoresed concurrently on one agarose gel that was subsequently probed with a mixture of radiolabeled *hgcp*-1, *gpcp*-1, or *gcp*-1 at the same time. Both *H. glycines* and *G. pallida* showed reduced cysteine proteinase transcript abundance, but only when octopamine was present with the dsRNA (Fig. 2A). As expected,

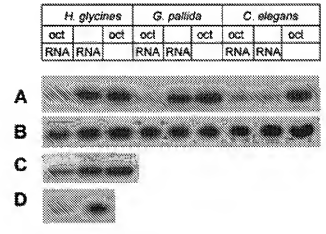

Fig. 2. Virtual Northern analysis of transcript abundance. Abundance of **A,** a cysteine proteinase; **B,** actin; **C,** *hgctl* transcript in preparasitic juvenile *Heterodera glycines* immediately after soaking; and **D,** MSP in male *Globodera pallida* first treated with dsRNA as juveniles prior to being used to infect plants. oct = the presence of octopamine; RNA = the presence of dsRNA in the soaking solution.

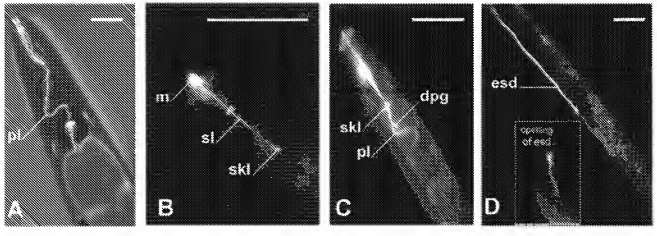

Fig. 1. Fluorescence microscopy showing ingestion of fluorescein isothiocyanate (FITC). **A,** After soaking in 1 mg of FITC per ml. FITC in the pharyngeal lumen (pl) of *Caenorhabditis elegans*. **B,** After soaking in 1 mg of FITC per ml and 10 mM octopamine, FITC in the lumen of the stylet of *Globodera pallida*. m = mouth opening, sl = stylet lumen, skl = lumen of the stylet knobs. **C,** FITC in the pharyngeal lumen of *Heterodera glycines*. dpg = duct of the dorsal pharyngeal gland. **D,** FITC in the length of the excretory/secretory duct (esd) of *H. glycines*. Inset: the opening of the excretory/secretory duct. No fluorescence was apparent in these structures when *G. pallida* and *H. glycines* were treated solely with FITC. Bar represents 20 μm.

the neurohumor was not required for *C. elegans* to show the effect, because the animal readily swallows the medium in which it is bathed. Stripping the virtual Northern blot and reprobing it with an actin probe confirmed equal loading of cDNA for all samples (Fig. 2B).

The second gene that was selected was a novel *H. glycines* gene (*hgctl*). It was chosen to explore the potential of RNAi to help determine if a gene of unknown function must be expressed for normal parasite development. This possibility is explored later, using J2 that had received RNAi treatments to challenge host plants. *hgctl* was isolated following monoclonal antibody (MAb) screening of a cDNA expression library (C. J. Lilley, *unpublished data*). *hgctl* is expressed most abundantly by adult female cyst nematodes during their period of rapid growth. It encodes a protein of 77 kDa with two C-type lectin (ctl) domains that are functional when expressed in *E. coli*. Its two lectin domains provide sequence homology with a cobra venom coagulation factor, macrophage mannose receptor, and proteoglycan core proteins such as aggrecan. *C. elegans* has a number of genes that are predicted to contain ctl domains, but none have high homology to *hgctl* (C. J. Lilley, *unpublished data*). RNAi suppressed the level of the *hgctl* transcript abundance in J2 (Fig. 2C).

The final transcripts we targeted for RNAi were those of the major sperm protein (MSP). MSP genes show a high degree of conservation, and sequence information on the five MSP genes of *G. pallida* (Novitski et al. 1993) was used to amplify the corresponding sequences from *H. glycines*. MSP genes were chosen for two reasons: any RNAi effect on these male-specific transcripts should not influence development of females, and secondly, MSP is only expressed in the sperm of males, which form more than 6 days after establishment of the J2 as a parasite (Wyss 1992). A pool of approximately 100 recovered male nematodes at 15 days postinfection (dpi) that had been treated with dsRNA as J2 did show a suppression of MSP transcript abundance (Fig. 2D). Therefore, an RNAi effect can be achieved for a cyst nematode gene that is first expressed several days after dsRNA treatment.

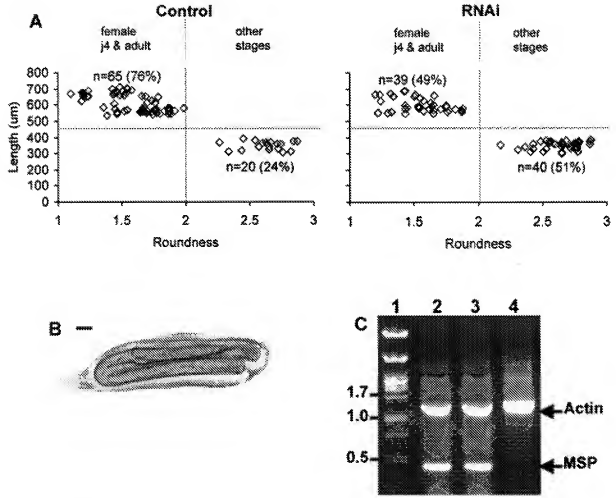

Fig. 3 Growth and sexual dimorphism of *Heterodera glycines* following RNAi of cysteine proteinases. **A,** The control represents animals treated with only octopamine and FITC; RNAi treatment represents animals treated with octopamine, fluoroscein isothiocyanate (FITC), and dsRNA of a cysteine proteinase. Filter values for roundness (R) and length (L) provide a basis to discriminate between developmental stages (Atkinson et al. 1996). At 14 days two groups are apparent: enlarged saccate animals, typically large feeding females (J4 and adult) and fusiform animals that may include females that are developmentally compromised, J2 animals that have not reached a stage of sexual differentiation, and males. **B,** Visual identification of fusiform *H. glycines* ($R > 2$; $L \leq 469$ μm) showed that nearly all had a vermiform shape inside the J3 cuticle, indicative of males. The remaining animals were developmentally less advanced but had the correct cuticular shape to suggest maleness. Bar = 20 μM. **C,** An example of amplification of major sperm protein (MSP) from individual animals using rtPCR. Lane 1, λ DNA restricted with *Pst*I; lane 2, fusiform animal, visually a mature male; lane 3, no visual sign of vermiform shape, but malelike cuticular shape; lane 4, cuticular shape less distinctive of a male. A band of the predicted size of MSP was apparent in lanes 2 and 3 but not in lane 4. A fragment of the correct size corresponding to actin (approximately 1.2 kb) was amplified in all the samples, indicating that polymerase chain reaction (PCR) had been successful. It should be noted that less developed males might not provide a PCR fragment representative of MSP.

Determination of postparasitic phenotype.

The consequence to subsequent plant parasitism of RNAi was studied for each of the three genes targeted at the J2 stage. Preparasitic juveniles of *H. glycines* and *G. pallida* were again stimulated to ingest and were exposed to dsRNA. RNAi treatment of cysteine proteinases of both cyst nematodes and the C-type lectin domain protein of *H. glycines* had an effect on subsequent development as parasites. After RNAi of cysteine proteinases, there was no decline in the number of nematodes established at 14 dpi, and normal growth rates were observed. However, the sexual fate of some individuals was changed. The proportion of *H. glycines* developing as females declined significantly from the expected value of 75% on controls to 50% after RNAi of the cysteine proteinase ($P < 0.001$) (Fig. 3A, Table 1). The same effect occurred for *G. pallida* with a fall from 77% in the control population to 56% ($P < 0.01$) for RNAi-treated animals (Table 1). New males have a distinct appearance that allowed visual identification to confirm that filter values used in image analysis correctly defined the sex of an animal (Fig. 3B). In addition, reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect MSP in individual animals, some of which were not as advanced in development as the animal shown in Figure 3B (Fig. 3C). Improved confidence in determination of males helped establish that changes in sexual fate did occur. In a further study involving RNAi of cysteine proteinases, nematodes recovered from plants 21 dpi showed a 40% reduction in the number of females relative to that in the controls ($P < 0.05$) (Table 1). This is consistent with a larger proportion of males for the RNAi treated population. Most males leave the plant by 21 dpi, whereas females are still sedentary parasites. At both 14 and 21 dpi there was no appreciable difference in the size of the females treated as juveniles with dsRNA corresponding to cysteine proteinases and that of the control females. In contrast, dsRNA directed at the transcript of *hgctl* did not affect the sexual fate of the nematodes, but it did reduce the number of parasites at 14 dpi by 41% ($P < 0.01$) (Table 1). The reduction in parasite numbers represents a failure of the nematodes to establish on the host plant. The results detect effects on either sexual fate or parasitic establishment arising from RNAi-targeted inhibition of gene expression. Neither effect occurred following RNAi of MSP, but males showed reduced transcript abundance of the corresponding transcript several days later.

DISCUSSION

While the current procedure already provides a basis for applying dsRNA to cyst nematodes, the inclusion of 3 mM spermidine and 0.05% gelatin in the soaking solution (Maeda et al. 2001) may improve the penetrance of molecules such as dsRNA and FITC. Phenotypes obtained by soaking and feeding have been considered weaker than those obtained by injection of *C. elegans* (Kuwabara and Coulson 2000; Tabara et al. 1998). However, ingestion of bacteria transcribing dsRNA has been shown to be at least as effective as microinjection. Embryonic lethal phenotypes are detected with similar efficiency with ingestion, leading to the detection of 50% more postembryonic phenotypes (Kamath et al. 2000). Soaking has been shown to have >97% penetrance for a number of genes whose loss of phenotype was well documented (Maeda et al. 2001). *C. elegans* postgenomic studies have also utilized high-throughput soaking RNAi protocols (Gonczy et al. 2000, Maeda et al. 2000).

The three initial genes to be targeted by RNAi were selected to show the power of RNAi in revealing the importance of gene function to parasitism. Biochemical inhibition of cysteine proteinases has revealed a dependence of cyst nematodes on this class of enzyme (Urwin et al. 1997a) and was therefore selected for the first demonstration of RNAi experiments. The recently identified c-type lectin gene was targeted by RNAi to determine if the importance for plant-nematode interactions could be addressed and whether RNAi could be used as an indication for prioritizing further analysis of genes. Finally, we analyzed MSP in an attempt to determine the period over which RNAi could have an effect. Transcriptional analysis of MSP following RNAi treatment establishes that the RNAi effect persists for several days, therefore the technique has the potential to treat J2s and then to study those genes first expressed only after the nematodes become established parasites. In *C. elegans*, RNAi effects can persist throughout an entire life cycle of the progeny of the injected animals (Kuwabara and Coulson 2000); however, the *C. elegans* life cycle is shorter than the duration of the RNAi effect described here for cyst nematodes.

RNAi is a sensitive technique, as dsRNA has a surprising ability to cross cellular boundaries, and the mode of action appears to involve an amplification of the original signal (Fire et al. 1998). All plant parasitic nematodes are obligate parasites. For cyst and other sedentary endoparasites, the invasive juveniles do not ingest and feed only as a postparasitic animal following formation of specialized feeding structures in the plant. It is possible that dsRNA can be delivered in planta to the nematode, providing the dsRNA is not excluded on a size basis, as occurs for dextrans (Bockenhoff et al. 1994) and proteins (Urwin et al. 1998). RNAi has been applied to plants, e.g., *Arabidopsis thaliana* (Chuang and Meyerowitz, 2000), and targeted at plant genes. RNAi may also be a basis for disrupting plant-nematode interactions. The application of RNAi described here will help improve understanding of genes involved in the interaction of cyst nematodes with their host.

Table 1. Number of females (J4 and adult) and all other stages collected from plants post-RNAi[a]

			Number of parasites					
			Female[b]		Other stages[c]		*P* value	
RNAi Target	Species	dpi	RNAi	Control	RNAi	Control	Ratio female/others	Total nematode number
Cysteine proteinase	*Heterodera glycines*	14	39	65	40	20	<0.001	NS
Cysteine proteinase	*H. glycines*	21	32	53	8	*	NS	<0.05
Cysteine proteinase	*Globodera pallida*	14	48	61	37	18	<0.01	NS
C-type lectin	*H. glycines*	14	32	54	14	24	NS	<0.01
MSP	*G. pallida*	14	41	34	19	20	NS	NS

[a] *P* values are for χ^2 test RNAi treatments relative to control; * No animals would be expected in this group at 21 dpi; sexual development is complete and males have left the plant.

[b] J4 and adult female.

[c] J2, J3, and adult male.

MATERIAL AND METHODS

RNA synthesis.

Full-length cDNA clones encoding a cysteine proteinase from both *H. glycines* (*hgcp*-I) (Urwin et al. 1997b) and its homologue from *G. pallida* (*gpcp*-I, isolated by screening the *G. pallida* cDNA library with *hgcp*-I) (C. J. Lilley, *unpublished data*) were available in the phagemid pBluescript. The coding region of the cysteine proteinase gene of *C. elegans*, *gcp-1* (Ray and McKerrow 1992), was also available in pBluescript (Urwin et al. 1995). *hgctl* (GenBank accession number AF498244) was isolated by screening a cDNA expression library (Lilley et al. 1997) with a monoclonal antibody (N46C10) as described by Sambrook and associates (1989). The MAb is one of many hundreds originally obtained using a shotgun approach with immunogens of homogenized females of *H. glycines* (Atkinson et al. 1988). Approximately 440 bp of the MSP open reading frame was amplified using the primers 5′AATTAACCCTCACTAAAGGGATGGCGCAACTTCCTC 3′ and 5′TAATACGACTCACTATAGGGACGTTGTACTCC-GATCGGCAAG3′ that incorporate the RNA primer sites T3 and T7 (underlined), respectively. The vectors harboring *hgcp*-I and *gpcp*-I were linearized with *Xho*I or *Eco*RI, the vector harboring *gcp*-I was linearized with *Sal*I or *Pst*I, the vector harboring *hgctl* was linearized with *Sac*I or *Kpn*I when driving transcription from the T3 or T7 promoters, respectively. To produce both sense and antisense RNA strands, in vitro transcription using the T3 and T7 promoters was carried out as specified by the manufacturer (Megascribe; Ambion, Oxfordshire, U.K.).

RNAi by soaking.

Preparasitic J2 *G. pallida* and *H. glycines* were collected from sterilized cysts as described by Urwin and associates (1995). *G. pallida* were hatched at 20°C, and *H. glycines* were hatched at 25°C. Approximately 10,000 animals were soaked in 50 mM octopamine made up in M9 salts (43.6 M Na_2HPO_4, 22 mM KH_2PO_4, 2.1 mM NaCl, 4.7 mM NH_4Cl) together with 1 mg FITC Isomer I (Sigma, Poole Dorset, U.K.) per ml (stock made up at 20 mg of FITC per ml DMF) and between 2 and 5 mg dsRNA per ml. Nematodes used as a control were soaked in only FITC and octopamine. The animals were left at room temperature for 4 h before being collected by brief microcentrifugation and then washed copiously with water to remove any external exogenous dsRNA. Uptake of FITC was viewed by fluorescence using a Leica microscope (model DMRB) with suitable filters. We found it beneficial to sort (done manually on the slide) animals showing uptake of FITC in the lumen of the stylet, pharynx, or in the excretory/secretory duct. Images were captured with a software package (Quantimet 500c; Leica, Cambridge) through either a color (Kappa CF15 MCC) or a black and white (COHU) camera mounted on the microscope.

Analysis of transcript abundance.

Approximately 10,000 preparasitic J2 animals were stimulated to ingest at any one time. Those showing FITC uptake were collected to analyze transcript abundance. PolyA+ RNA was isolated from J2 animals using a QuickPrep Micro mRNA purification kit (Amersham Pharmacia, Bucks, U.K.). Transcript abundance was determined by virtual Northern analysis that utilizes SMART PCR cDNA synthesis technology (Clontech, Hampshire, U.K.). A modified oligonucleotide (dT) primer was used to prime the synthesis of first strand cDNA. At the 5′ end of the mRNA, the terminal transferase activity of reverse transcriptase adds a few cytidine residues at the 3′ end of the first strand cDNA. An oligonucleotide primer with polyG sequence at its 3′ end hybridizes to the polyC sequence and extends the template. The reverse transcriptase switches template and continues replicating to the end of the oligonucleotide. The oligonucleotide sequences at the 3′ and 5′ ends are then used to amplify cDNA by long-distance PCR; the number of cycles is determined empirically for each experiment but is often 18. The cDNA is then size-fractionated by agarose electrophoresis, transferred to an immobilized support, and probed with a suitably radiolabeled-DNA sequence using standard protocols (Sambrook et al. 1989).

Infection of plants.

FITC-positive J2 animals were used to infect plants grown in pouches, as described by Atkinson and Harris (1989). Commonly, between four and six infection points were established on the host plant, with approximately 60 nematodes being used at each infection point. The percentage of infection was about 10% for both the controls and for those nematodes treated with dsRNA. Two plants, either *Solanum tuberosum* spp. *tuberosum* cv. Desiree or *Glycines max* were placed in a pouch and infected with *G. pallida* Pa2/3 or *Heterodera glycines*, respectively. Pouches (8 to 10) were set up for both the treatment, nematodes exposed to octopamine, FITC, and dsRNA, and the control groups, nematodes exposed to only octopamine and FITC. Studies on all genes targeted by RNAi were replicated.

Observation of phenotypes.

Animals were collected from the roots after 14 or 21 dpi and studied by image analysis (Atkinson et al. 1996). Presumptive males were subject to RT-PCR using an ABgene kit (ABgene, Surry, U.K.). Individual animals were first dissected from root material and ground in a microcentrifuge tube. Hybaid Recovery amplification reagent was used as described by the manufacturer (Hybaid, Middlesex, U.K.). Primers were designed against the published sequence of MSP (Novitski et al. 1989) with the sequence 5′ATGGCGCAACTTCTTCC3′ and 5′ACG-TTGTACTCGATCGGCAAG3′. As a control in RT-PCR, primers were designed to amplify an actin from *G. pallida*, 5′AGTACCCGATTGAGCACGGC3′ and 5′GGCGAATGGG-TCGGCGGATGG3′. It was possible to use all the primers together in the same standard RT-PCR reaction.

ACKNOWLEDGMENTS

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LITERATURE CITED

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ORIGINAL ARTICLE

Host-delivered RNAi: an effective strategy to silence genes in plant parasitic nematodes

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Abstract Root-knot nematodes (*Meloidogyne* spp.) are obligate, sedentary endoparasites that infect many plant species causing large economic losses worldwide. Available nematicides are being banned due to their toxicity or ozone-depleting properties and alternative control strategies are urgently required. We have produced transgenic tobacco (*Nicotiana tabacum*) plants expressing different dsRNA hairpin structures targeting a root-knot nematode (*Meloidogyne javanica*) putative transcription factor, *MjTis11*. We provide evidence that *MjTis11* was consistently silenced in nematodes feeding on the roots of transgenic plants. The observed silencing was specific for *MjTis11*, with other sequence-unrelated genes being unaffected in the nematodes. Those transgenic plants able to induce silencing of *MjTis11*, also showed the presence of small interfering RNAs. Even though down-regulation of *MjTis11* did not result in a lethal phenotype, this study demonstrates the feasibility of silencing root-knot nematode genes by expressing dsRNA in the host plant. Host-delivered RNA interference-triggered (HD-RNAi) silencing of parasite genes provides a novel disease resistance strategy with wide biotechnological applications. The potential of HD-RNAi is not restricted to parasitic nematodes but could be adapted to control other plant-feeding pests.

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Abbreviations

dsRNA	Double-stranded RNA
HD-RNAi	Host-delivered RNA interference
RKN	Root knot nematode
RNAi	RNA interference
qRT-PCR	Quantitative real time PCR
siRNA	Small interfering RNA

Introduction

Parasitism of plants by nematode infestation causes extensive damage to turf grasses, ornamental plants and food crops each year. Over 20% of the annual yield losses in major crops are caused by plant-parasitic nematodes with an economic loss estimated to be in excess of US $ 77 billion worldwide (Jung and Wyss 1999). Most of the damage is caused by the sedentary endoparasitic nematodes of the Tylenchoidea superfamily . This superfamily contains the cyst nematodes (part of the Heteroderidae family) and the root-knot nematodes (part of the Meloidogynidae family). Symptoms of diseased plants include stunting, wilting and enhanced susceptibility to other diseases. Some cultivars from species such as tomato, potato and soybean contain nematode resistance genes that can be effective against several nematode species, but the protection is usually quite narrow, controlling only specific pathotypes of a species. There are also many crops for which resistance loci are not available (e.g. sugar beet and pineapple). Root-knot nematodes (RKN) have the ability to infect thousands of plant species (Abad et al. 2003) making crop rotation of little value as a control strategy. Consequently, efforts to

eliminate or minimize damage caused by nematodes have typically involved the use of soil fumigation. Although the use of nematicides such as methyl bromide, organophosphates and carbamates is effective in reducing nematode population levels, their cost and toxicity to humans and the environment make them unsound as a control measure in plant production. Indeed, the use of many available nematicides is being banned due to their toxicity or ozone-depleting properties, and alternative control strategies are urgently required.

Plant root-knot nematodes hatch as motile and non-feeding second-stage juveniles (J2). They are attracted to root tips, where they penetrate the epidermis in the elongation zone and migrate intercellularly towards the stele. RKNs become sedentary after establishing a permanent feeding site in surrounding phloem parenchyma cells by stimulating and maintaining giant cell formation (Jung and Wyss 1999; Gheysen and Fenoll 2002; Abad et al. 2003; Williamson and Gleason 2003; Davis et al. 2004). These cells are multinucleated and undergo extensive remodelling of the cell wall, adopting characteristics of transfer cells. Giant cells act as sinks, diverting plant nutrients to provide metabolic energy for the nematode that feeds exclusively from them. Concurrently with giant cell formation, nearby pericycle and cortical cells enlarge and divide forming the root-knot or gall that is characteristic of infection by this group of nematodes.

The discovery that feeding dsRNA to the nematode *Caenorhabditis elegans* inactivates endogenous genes (Timmons and Fire 1998; Timmons et al. 2001) by an RNA interference (RNAi) mechanism (Fire et al. 1998; Hannon 2002) suggests that a similar mechanism could also be present in other nematode species. We therefore devised a strategy to inactivate nematode genes using giant cells as delivery vehicles to provide dsRNA molecules to the feeding nematode. For such a strategy to be successful, root-knot nematodes must have a similar RNAi response as the distantly related free-living bacteriovore *C. elegans*. Recent work has demonstrated such a response after soaking J2 juveniles of potato and soybean cyst nematodes (Urwin et al. 2002; Chen et al. 2005) as well as the RKN *M. incognita* (Bakhetia et al. 2005a; Rosso et al. 2005) in a solution containing dsRNA. Also recently, a number of manuscripts have appeared using different RNAi-based approaches to control nematode infestation in plants (Huang et al. 2006; Steeves et al. 2006; Yadav et al. 2006; Gheysen and Vanholme 2007).

The aim of this study is to demonstrate the feasibility of down-regulating endogenous root-knot nematode transcripts by expressing dsRNA with sequence identity to the nematode gene in plant roots. We also study whether the silencing is sequence-specific, affecting only the targeted gene and not other unrelated nematode genes.

Materials and methods

Construction of plasmids

Meloidogyne javanica MjTis11 EST clone (rk10c12.y1, GenBank accession BE578298) was obtained from the Washington University Parasitic Nematode EST Project (McCarter et al. 2000). Complete DNA sequencing of the clone was carried out and sequence analysed using the University of Wisconsin Genetics Computer Group (UWGCG) software version 8.1 (Devereux et al. 1984).

RNAi constructs were prepared by adding appropriate restriction sites to the ends of the primers used to perform PCR amplification with Elongase DNA polymerase (Invitrogen) as described by Chakravorty and Botella (2007), followed by subcloning into the vector pHannibal (Wesley et al. 2001, for the intron containing hairpin structures) or a derivative of this vector that had the intron replaced with a fragment of the *uidA* gene of the same size (741 bp). In all cases, PCR-amplified DNA sequences were sequenced to confirm that no errors had been introduced during amplification. The expression cassette from pHannibal was then subcloned into the binary vector pUQC477 (Fig. 3). Binary vectors were transferred by triparental mating into *Agrobacterium tumefaciens* LBA4404 (Hoekema et al. 1983), which was then used for plant transformation.

Plant transformation

The rapid flowering tobacco (*Nicotiana tabacum* L.) variety Ti68 (McDaniel et al. 1996) was transformed and regenerated according to a modified leaf-disk method (Horsch et al. 1985). Independent primary transgenic lines were propagated in tissue culture and rooted clonal replicates transferred to soil and acclimatized in a growth cabinet before transfer to the glasshouse. Possible effects of tissue culture on nematode feeding were addressed by producing control lines (TisA) through normal transformation procedures.

Nematode challenge

Eggs of *M. javanica* were recovered from stock cultures maintained on tomato plants. Regenerated transgenic tobacco plants were transferred to 400 ml pots containing sand-based compost and osmocote nutrient granules. Two weeks after potting, the plants were inoculated using 10,000 *M. javanica* eggs and grown in a glasshouse. For each experiment, plants were positioned in a randomized way in glasshouse benches and infected with the same batch of nematode eggs. Plants were harvested 6 weeks after inoculation. Depending on the experiment, a total of 13–16 independent transgenic lines were analysed.

Molecular analyses

At the time of collection, 6 weeks after inoculation, all roots were heavily infected (level 5). Roots from transgenic 35S promoter, TobRB7 promoter and WT plants were briefly bleached during harvesting to remove nematode eggs and newly hatched J2 juveniles. The majority of nematodes left inside the root tissues were mature females and late stage females. Acid fuschin staining confirmed that these stages were the predominant type in the harvested roots. Whole roots were used for cDNA production; therefore, the cDNA pool contained nematode and tobacco root cDNAs. However, nematode cDNAs were relatively abundant in the sample as supported by the Ct value for nematode actin being approximately 25.

For real-time quantitative PCR experiments (qRT-PCR), 1 μg of total RNA was reverse transcribed using Superscript III (Invitrogen) following the manufacturer's instructions. The resulting cDNA was subsequently diluted to a total volume of 250 μL. *M. javanica* target genes were amplified using gene-specific primers for *MjTis11* (forward, 5′-CTTGGGTTTAATTACCCAAGTTTGAGAT-3′ and reverse, 5′-TCCACGCGGACAATAACCTTTA-3′), *MjGAPDH*, coding for glyceraldehydes-3-phosphate dehydrogenase (forward, 5′-TGCTTCCTGCACTACTAACTG TCTTG-3′ and reverse, 5′-CAGTAACAGCGTGCACAG TAGTCAT-3′), *Mjα-tubulin* (forward, 5′-CCGACAACT GCCAAGGACTT-3′ and reverse, 5′-CAACCGAAAGAC GCTCCATAA-3′) and *Mjβ-actin* (forward, 5′-TGTCAAT GTCGCACTTCATGATC-3′ and reverse, 5′-CCGTTGC CCAGAATCTCTCTT-3′) designed using Primer Express 1.5 software (Applied Biosystems, Foster City, CA, USA) and consensus sequences of *Mjα-tubulin* and *Mjβ-actin* assembled from several public expressed sequence tags. qRT-PCR was carried out as described by Moyle et al. (2005) using the ABI PRISM 7700 sequence detector and SYBR Green Master mix (Applied Biosystems) using primers at a final concentration of 0.1 μM each and 2.5 μL (the equivalent of 10 or 0.1 ng total RNA) of cDNA as template. PCR-cycling conditions comprised an initial polymerase activation step at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Real-time DNA amplification was monitored and analysed using the Sequence Detector 1.7 software (Applied Biosystems). Quantification of gene expression relative to the β-actin reference gene was calculated using the Pfaffl equation (Pfaffl 2001), the amplification rate and cycle threshold (Ct). This equation takes into account differing primer efficiencies. Cycle threshold values were corrected for genomic DNA contamination in the RNA sample. An infected wild-type root sample, referred to as WTCAL in the text, was used as a calibrator to allow comparisons across samples.

For siRNA detection, total RNA was extracted from 2–3 g of ground leaf tissue using TRIzol reagent (Life Technologies/Gibco BRL) and a extraction buffer containing 0.5% SDS, 1 M NaCl, 1 mM EDTA, 10 mM Tris–HCl, pH8.0. High molecular weight RNAs (mRNA and rRNA) were precipitated by adding 1 volume 20% PEG (MW 8000), 1 M NaCl, incubating at 4°C for 30 min and centrifuging for 30 min at room temperature. Low molecular weight (LMW) RNAs including siRNAs were ethanol precipitated from the PEG/NaCl supernatant by adding 3 volumes of ethanol and centrifuging for 30 min at 4°C. After washing the pellet with 80% ethanol, an aliquot was redissolved in water for quantification and the remainder redissolved in formamide for gel electrophoresis. Purified LMW RNAs (2–10 μg) were separated by denaturing (7 M urea) polyacrylamide (15%, 19:1) gel electrophoresis in 1 × tris borate EDTA buffer. The separated LMW RNAs were transferred to Hybond NX membrane (Amersham) by electroblotting. Antisense riboprobes were prepared from linearized plasmids containing the *MjTis11* or *uidA* gene with 5′[α-^{32}P] rCTP using SP6 RNA polymerase and the "Riboprobe" system from Promega as per manufacturer's instructions. Hybridization conditions against the blotted LMW RNAs were performed as described by Trusov et al. (2006). All hybridization signals were detected by phosphorimaging as described by Purnell et al. (2005).

Statistical analysis

JMP IN 4.0 software (SAS Institute Inc.) was used for statistical analysis of the qRT-PCR data. The normality assumption was tested using a normal quantile plot and a Shapiro-Wilk W goodness of fit test. Each set of independent transgenic plants had a P value of 0.03 or less, indicating a normal distribution. The Student's t test with an alpha level of 0.05 was used to compare relative transcript level means. For the calculation of the t test, all data from nematodes feeding on a specific genotype (such as TisS plants) was considered together.

Results

MjTisII is mainly expressed at the egg and mature female stages

Functional genomic screens of *C. elegans* using dsRNA feeding techniques have shown that most genes resulting in a lethal phenotype are highly expressed in embryos rather than adult tissues (Fraser et al. 2000). We chose a *M. javanica* Tis11 zinc finger $CX_8CX_5CX_3H$ type putative transcription factor (*MjTis11*, GenBank #BE578298) that is mainly expressed in eggs and in egg-producing adult females (Fig. 1). *In-silico* studies showed that MjTIS11 has limited similarity to the *C. elegans* MEX-1, PIE-1 and

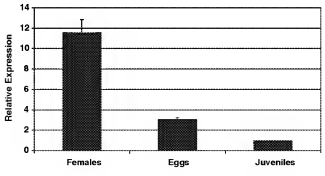

Fig. 1 Expression levels of *MjTis11* in *M. javanica*. Real-time qRT-PCR analysis of *M. javanica* putative transcription factor *MjTis11* transcript levels at different stages of the nematode life cycle. *MjTis11* mRNA abundance was normalized to actin levels and shown relative to mRNA levels at the juvenile stage. Each *bar* represents the mean of duplicate assays repeated twice. Standard errors are shown for females and eggs, but not for juveniles since mRNA levels are shown relative to this stage. All differences are statistically significant ($P < 0.001$)

POS-1 proteins (Fig. 2), which are involved in determining cell fate during the early stages of embryogenesis. RNAi-mediated silencing of these genes result in an embryo lethal phenotype in *C. elegans* (http://www.wormbase.org). Extensive database searches showed that *MjTis11* has no apparent homology with any known plant genes, thereby minimizing any potential RNAi off-target effects (Bakhetia et al. 2005b). In addition, PCR experiments using the specific primers designed for *MjTis11* failed to amplify any product using either tobacco cDNA samples from several tissues or tobacco genomic DNA (results not shown).

Reproducible down-regulation of *MjTisII* in nematodes feeding on transgenic tobacco roots

A total of four RNAi constructs were prepared for plant transformation (Fig. 3). Two of these consisted of an inverted repeat of a 772 bp fragment of the *MjTis11* cDNA, separated by either a spacer region (TisS) or an intron (TisI), and driven by the constitutive 35S cauliflower mosaic virus promoter (CaMV35S). The remaining two also contained an inverted repeat of the *MjTis11* cDNA fragment, but driven by the gall-specific Δ0.3 TobRB7 promoter (TisTS and TisTI; Opperman et al. 1994). A control construct (TisA) was also prepared containing the *MjTis11* cDNA in antisense orientation driven by the CaMV35S promoter.

In order to monitor endogenous *MjTis11* transcript levels in *M. javanica* nematodes, we used real-time quantitative PCR (qRT-PCR) with primers designed towards a 5′ region of *MjTis11* (*5′MjTis11*), upstream of the fragment used in our binary constructs. This allowed the endogenous *MjTis11* transcript levels in nematode tissues to be monitored independently of any *MjTis11* transcripts present in transgenic plant tissues (originating from the transcription of the inserted T-DNA). *5′MjTis11* levels were normalized to *M. javanica* actin (GenBank #AF532604) levels in order

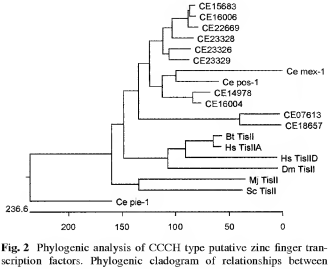

Fig. 2 Phylogenic analysis of CCCH type putative zinc finger transcription factors. Phylogenic cladogram of relationships between CCCH type putative zinc finger transcription factors from *C. elegans* [WormBase protein IDs CE15683, CE16006, CE22669, CE23328, CE23326, CE23329, CE14978, CE16004, CE07613, CE18657, CE28097 (Ce mex-1), CE04629 (Ce pos-1), CE28134 (Ce pie-1)], cow [Bt TisII (GenPept #P53781)], human [Hs TisII, Hs TisIID (GenPept #P26651 and #P47974, respectively)], fly [Dm TisII (GenPept #P47980)], yeast [Sc TisII (GenPept #NP_013237)] and *Meloidogyne javanica* (Mj TisII). Branch distances are proportional to sequence divergence

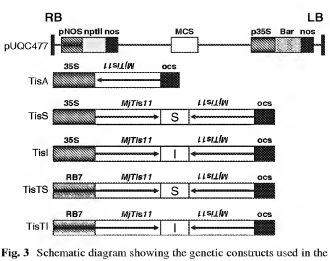

Fig. 3 Schematic diagram showing the genetic constructs used in the production of transgenic tobacco plants. Schematic diagram of the different T-DNA cassettes used to produce the transgenic tobacco lines used in this study. The T-DNA components were the nopaline synthase promoter (*pNOS*) and terminator (*nos*), CaMV35S promoter (*35S*), Δ0.3 TobRB7 promoter (*RB7*), neomycin phosphatase (*nptII*), pdk intron (*I*), spacer region (*S*), phosphinothricin acetyltransferase (*bar*), octopine synthase terminator (*ocs*) and *MjTis11*. The five different cassettes TisA, TisS, TisI, TisTS and TisTI were cloned into the multiple cloning site (*MCS*) of the binary vector pUQC477

to compare data from different samples. In this way, the relative expression levels of *MjTis11* in nematodes could be determined independent of the severity of nematode infection or the initial amount of nematode tissue present in each sample. To ensure that the primers used did not result in non-specific amplification of a tobacco endogenous gene, PCR experiments were conducted with tobacco cDNA and genomic DNA with negative results.

In preliminary screenings, samples from infected roots of 13–15 independent transgenic lines for each of the five constructs as well as wild type (WT) were analysed. Our results showed a decrease in *5′MjTis11* transcript levels in nematodes feeding on the roots of transgenic tobacco lines expressing dsRNA *MjTis11* under the control of the CaMV35S promoter (lines TisS and TisI; Fig. 4a). None of the lines containing the TobRB7 promoter showed signs of *MjTis11* silencing. We have produced transgenic tobacco lines carrying the β-glucuronidase (GUS) reporter gene under the control of the Δ0.3 TobRB7 promoter. Detailed analysis of several transgenic lines showed that (1) the majority of galls displayed no observable GUS staining, and (2) for the small percentage of galls showing some GUS activity, the intensity of the stain was very light (results not shown). The lack of silencing observed in the TobRB7 promoter lines could therefore be attributed to the weakness of the TobRB7 promoter. Of the two constructs showing some silencing effect in nematodes, the RNAi construct with the spacer (TisS) was much more effective than the construct containing the intron (TisI). This was somewhat surprising since the use of an intron rather than a spacer has been reported to improve the efficiency of gene silencing (Smith et al. 2000).

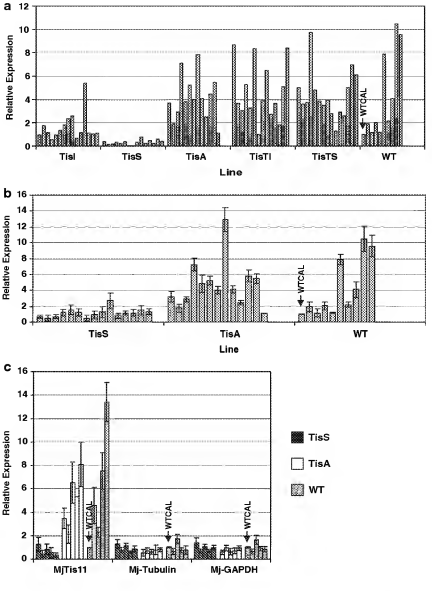

Fig. 4 Silencing of the *MjTis11* gene in nematodes feeding on transgenic plants is reproducible and dependent on the structure of the introduced construct. Real-time qRT-PCR was used to determine mRNA abundance of *M. javanica* genes from nematodes living in wild-type and transgenic plants from independent transgenic lines. mRNA abundance was normalized to actin levels and shown relative to mRNA levels in wild-type sample WTCAL. **a** Each *bar* represents the mean of duplicate assays for an individual WT plant or transgenic line. **b** Each *bar* represents the mean of duplicate assays repeated twice for three clonal replicates of the TisS lines and individual WT and TisA plants. **c** Expression levels of *MjTis11*, *Mjx-tubulin* (GenBank #BG736650) and *Mj-GAPDH* (GenBank #BM881590) in nematodes living in roots of selected TisS (*black*), TisA (*white*) and WT (*grey*) plants. Each *bar* represents the mean of duplicate assays repeated three times. *Error bars* representing standard error of the mean are shown

To verify the results from the preliminary screening, additional experiments were carried out focusing on the best performing construct (TisS) and using two different controls (TisA and WT). In this occasion, duplicate qRT-PCR determinations were performed on three biological replicates for each TisS line analysed. The results shown in Fig. 4b confirmed that nematodes feeding on transgenic plants carrying the TisS construct had significantly lower levels of *MjTis11* than nematodes feeding on either control TisA lines or WT plants (Student's *t* test, alpha value = 0.05). Relative expression levels for all the 15 TisS lines analysed were very consistent and similar to those obtained in the preliminary screen (Fig. 3a).

The down-regulation observed for *MjTis11* does not affect other nematode genes

Selected TisS lines were further examined to determine whether the decrease in transcript levels observed for *MjTis11* was specific for this gene or whether other sequence-unrelated genes were also affected in the nematodes. For this purpose, the expression levels of *MjTis11*, *Mjα-tubulin* and *MjGAPDH* in nematodes feeding on the roots of TisS lines, control TisA or WT plants were measured by qRT-PCR and normalized to nematode actin levels. As observed before (Fig. 4a, b), there was a significant (Student's *t* test, alpha value = 0.05) and reproducible reduction in *MjTis11* transcript levels in *M. javanica* feeding on the roots of TisS lines, when compared to nematodes feeding on control TisA and WT plants (Fig. 4c). In contrast, the levels of *Mjα-tubulin* and *MjGAPDH* were not significantly different among the three groups of plants studied, suggesting that the down-regulation observed for *MjTis11* is sequence-specific. It is important to note that in Fig. 4c, *MjTis11* levels are only comparable among the three populations of plants (TisS, TisA and WT), but are not comparable with the levels observed for *Mjα-tubulin* and *MjGAPDH*.

Down-regulation of *MjTisII* is associated with the existence of small interfering RNAs in the transgenic tobacco line

In order to determine whether the transgenic lines causing down-regulation of *MjTis11* in nematodes were producing small interfering RNAs (siRNAs) with homology to the *MjTis11* dsRNA, we analysed samples from TisS, TisI, TisA and WT plants. Leaves of TisS and TisI plants, but not TisA and non-transgenic controls, showed the presence of siRNAs (Fig. 5). To provide positive and negative controls for siRNA detection, transgenic tobacco plants expressing the *β*-glucuronidase (*GUS*) gene were retransformed with a *GUS* hairpin construct. A number of lines showed silencing of the *GUS* gene, while in others no

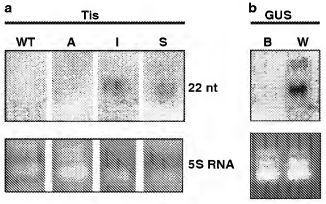

Fig. 5 Silencing of the *MjTis11* gene is specific and associated with the presence of siRNAs. **a** RNA gel-blot showing the accumulation of *MjTis11* siRNAs in TisS (*lane S*) and TisI (*lane I*) plants, but not in TisA (*lane A*) and WT controls. **b** RNA gel-blot showing the accumulation of *GUS* siRNAs in silenced (*lane W*) and non-silenced (*lane B*) GUS transgenic tobacco plants expressing a GUS hairpin construct. Ethidium bromide stained gels are shown as loading controls

silencing was observed. The presence of *GUS* siRNAs was detected in *GUS* silenced, but not in non-silenced plants (Fig. 5). Although not directly comparable, it appears that the levels of siRNAs were lower in the *MjTis11* plants than those observed in the leaves of *GUS* silenced plants.

Discussion

Previous studies have shown that it is possible to achieve an RNAi-like response in cyst and RKN nematodes (Urwin et al. 2002; Bakhetia et al. 2005a; Chen et al. 2005; Rosso et al. 2005). Nevertheless, these nematodes do not feed until they are established inside their host plants; therefore, the above-mentioned studies induced the nematodes to ingest dsRNA by octopamine or resorcinol treatment. The silencing achieved was transient with normal transcript levels returning 68 h after soaking (Rosso et al. 2005). A key question for the potential application of HD-RNAi was whether nematodes can ingest dsRNA when feeding on plant cells expressing hairpin constructs. Root-knot and cyst nematodes produce specialized structures inside the cells on which they feed called feeding tubes (Hussey and Mims 1991). These act as molecular sieves and may help to protect the nematode from ingesting plant defence macromolecules. The size exclusion limit is unknown, but experiments using GFP (28 kDa) and different dyes indicate a size exclusion between 20 and 40 kDa (Bockenhoff and Grundler 1994; Urwin et al. 1997). It has been suggested that linear dsRNA as well as double-stranded small interfering RNAs (siRNAs) of 21–23 nucleotides could be taken up lengthwise by *M. incognita* (Bakhetia et al. 2005b). The siRNAs are produced from long dsRNA molecules by an

RNase III-like ribonuclease before being incorporated into an RNA-induced silencing complex (RISC) that identifies and degrades mRNAs showing homology to the dsRNA. However, the feeding tube would be expected to exclude both non-linear large dsRNA molecules and siRNAs incorporated into RISC (360 kDa; Nykanen et al. 2001).

The ultimate aim of our research was to devise methods to control nematode infestation in agriculturally important crops. The selection of the targeted gene (*MjTis11*) was based on the fact that RNAi-driven silencing of some homologous *C. elegans* genes resulted in an embryo lethal phenotype. Down-regulation of *MjTis11* transcript levels in *M. javanica* nematodes did not result in a significant decrease in fecundity or egg hatching rate in any of the lines analysed (results not shown), indicating that either (1) this gene is not a good candidate to achieve an embryo lethal phenotype in *M. javanica* and/or (2) the levels of down-regulation achieved for *MjTis11* are not sufficient to compromise its biological role. In fact, even though silencing of the *C. elegans* genes coding for the MjTIS11 homologous proteins MEX-1, POS-1 or PIE-1 resulted in an embryo lethal phenotype, silencing of ten other *C. elegans* TIS11 transcription factors, also homologous to MjTIS11 and clustering together in the phylogenetic tree (Fig. 2), produced a wild-type phenotype (http://www.wormbase.org). Therefore, for this particular family of transcription factors, identifying an *M. javanica* orthologue of an essential *C. elegans* gene in order to achieve an embryo lethal phenotype could be difficult based solely on *in-silico* analyses, as the protein similarity is confined to the zinc finger motifs. Regardless, the fact that partial silencing of *MjTis11* was obtained demonstrates that plants can be used as delivery systems to induce down-regulation of targeted genes in parasitic nematodes. High-throughput strategies can now be devised to find appropriate gene targets in *M. javanica*. During the production of this manuscript, a report by Yadav et al. (2006) has been published describing the use of hairpin constructs to control infestation of a different plant parasitic nematode, *Meloidogyne incognita* in tobacco. Down-regulation of the target genes selected by Yadav et al. (2006), an integrase and a splicing factor, protected the plant against infection by *M. incognita*. Even more recently, Huang et al. (2006) found that expression in *Arabidopsis thaliana* of a conserved RKN gene essential for plant parasitism resulted in broad range resistance against four major RKN species.

The large differences observed in *MjTis11* expression levels (Fig. 3a, b and MjTis11 in c) within each specific genotype, but most apparent in WT and non-silenced lines (TisA, TisTI and TisTA), could reflect the developmental stage of the nematode population present in the sample. We have shown that *MjTis11* expression levels can increase up to 12 times from juveniles to adult females; therefore, a particular sample enriched in adult females could result in increased levels of *MjTis11* message. In fact, this variation was not observed when more "developmentally constant genes" such as *Mjx-tubulin* and *MjGAPDH* were measured, proving that the difference in *MjTis11* were not due to experimental artifacts (Fig. 4c). It is important to note that *MjTis11* expression levels did not show such large variation in silenced TisS lines.

Our data indicated that the decrease in transcript levels observed for *MjTis11* was specific for this gene and did not affect two other unrelated nematode genes *Mjx-tubulin* and *MjGAPDH* (Fig. 4c). This suggests that (1) the silencing is caused by the transgene introduced in the host plants and (2) that the silencing is caused by an RNAi-like mechanism. Nevertheless, introduction of a hairpin-containing transgene is not sufficient to induce silencing unless it is linked to a strong promoter, as is shown by the absence of silencing observed in nematodes feeding on lines driven by the weak TobRB7 promoter (Fig. 3a). Another characteristic of RNAi is the production of siRNAs with homology to the dsRNA trigger, which was detected in transgenic lines. In plants, fungi and nematodes siRNAs can serve as primers for the synthesis of dsRNA by RNA-dependent RNA polymerases, producing secondary siRNAs by "transitive RNAi" and resulting in strong amplification of RNAi silencing (Lipardi et al. 2001; Sijen et al. 2001; Vaistij et al. 2002). However, no secondary siRNAs are expected to be produced in the TisS and TisI lines, since the targeted sequence is not present in the tobacco genome. Our data suggested that transgenic lines expressed relatively low levels of *MjTis11* siRNAs compared to those detected in GUS-silenced plants (Fig. 5). These observations provide a potential explanation why complete silencing of the nematode *MjTis11* gene was not achieved since it is believed that siRNAs must accumulate above a certain threshold for complete silencing to occur. In this respect, the simultaneous expression of the *MjTis11* gene and the *MjTis11* hairpin structure in root galls may boost the cellular levels of *MjTis11* siRNAs, resulting in more effective silencing of the nematode gene.

In summary, this paper describes the production of transgenic tobacco plants expressing dsRNAs targeting a root-knot nematode (*Meloidogyne javanica*) putative zinc finger transcription factor, *MjTis11*. We provide evidence that *MjTis11* was silenced in nematodes feeding on the roots of these transgenic plants and that the silencing probably resulted from an RNAi-like mechanism. Our results demonstrate that plants can be used as delivery systems to induce RNAi-mediated gene silencing in parasites such as root-knot nematodes.Host-delivered RNA interference (HD-RNAi) will allow the development of novel methods to control nematode infestation in plants. However, further research is needed to increase both the level of silencing

and to identify nematode genes whose silencing results in a lethal phenotype. The basic principle behind HD-RNAi is not restricted to nematode pests and, in theory, could also be applied to all those agricultural pests that feed on plant tissues and are susceptible to an RNAi-type gene silencing.

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